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AU Yamaguchi, Masamitsu; Hayashi, Yuko; Matsuoka, Shuhei; Takahashi, Takashi; Matsukage, Akio
SO European Journal of Biochemistry (1994), 221(1), 227-37
What promoter drives p53 expr??

TI Wild-type human p53 transactivates the human proliferating cell nuclear antigen promoter
AU Shivakumar, Chittari V.; Brown, Doris R.; Deb, Sumitra; Deb, Swati Palit
SO Molecular and Cellular Biology (1995), 15(12), 6785-93
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# Inhibition of Viral and Cellular Promoters by Human Wild-Type p53

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**Mutation of the p53 tumor suppressor gene is a recurring event in a variety of human cancers. Wild-type p53 may regulate cell proliferation and has recently been shown to repress transcription from several cellular promoters. We studied the effects of wild-type and mutant human p53 on the human proliferating-cell nuclear antigen promoter and on several viral promoters including the simian virus 40 early promoter-enhancer, the herpes simplex virus type 1 thymidine kinase and UL9 promoters, the human cytomegalovirus major immediate-early promoter-enhancer, and the long terminal repeat promoters of Rous sarcoma virus, human immunodeficiency virus type 1, and human T-cell lymphotropic virus type I. HeLa cells were cotransfected with a wild-type or mutant p53 expression vector and plasmids containing a chloramphenicol acetyltransferase reporter gene under viral (or cellular) promoter control. Expression of wild-type p53 correlated with a consistent and significant (6- to 76-fold) reduction of reporter enzyme activity. A mutation at amino acid 143 of p53 releases this inhibition significantly with all the promoters studied. Expression of a p53 mutated at any one of the five amino acid positions 143, 175, 248, 273, and 281 also correlated with a much smaller (one- to sixfold) reduction of reporter enzyme activity from the herpes simplex virus type 1 thymidine kinase promoter. These mutant forms of p53 are found in various cancer cells. Thus, failure of tumor suppression correlates with loss of the promoter inhibitory effect of p53.**

p53 is a nuclear phosphoprotein that was initially detected in association with simian virus 40 (SV40) large T antigen in virus-transformed rodent cells (31, 33). Elevated levels of p53 were subsequently observed in cell lines transformed by a variety of agents, including DNA and RNA tumor viruses, irradiation, and chemical carcinogens (13, 16, 24, 29, 35, 48). When genomic and cDNA clones of p53 were found to immortalize primary cells and to cooperate with the *ras* oncogene in transformation of primary cells, p53 was consigned to the nuclear oncogene family of *myc* and *myb* (16, 29, 45); only recently has it been learned that the original clones contained activating mutations (24). Expression of wild-type p53 has now been shown to inhibit proliferation of transformed cells, suppress oncogene-mediated cell transformation, and eliminate the tumorigenic potential of tumor-derived cell lines (2, 3, 8, 9, 14–16, 18, 36, 38, 40). Like the retinoblastoma susceptibility (RB) gene, p53 is now considered to be an antioncogene or tumor suppressor gene (see reference 32 for a review). Somatic and germ line (in Li-Fraumeni syndrome) mutation of the p53 gene has been detected in a variety of human tumors, with mutations concentrated in phylogenetically conserved sequence domains (26, 32, 34, 54). At present, p53 mutations are the most frequently reported genetic defects in human cancer (3, 26, 27, 44, 56, 58).

Several biochemical functions are attributed to p53. p53-GAL4 fusion proteins can activate transcription from promoters containing GAL4-binding sites, suggesting that p53 is a transactivator (17, 47). Moreover, sequence-specific DNA binding by p53 has been reported (4, 30). Wild-type (but not mutant) p53 binds to the 21-bp repeats of the SV40 early and late promoters (4) and to TGCCT repeats present in the human ribosomal gene cluster (30). p53 inhibits SV40 DNA replication in vivo and in vitro by complexing with T antigen and inhibiting the unwinding capability of T antigen (5, 19, 20, 59). Wild-type p53 has recently been shown to inhibit c-*fos* transcription (21) and to repress transcription from several cellular promoters (10, 21, 50).

The human proliferating-cell nuclear antigen (PCNA) gene is growth regulated (1, 28) and encodes a protein that is a component of the DNA replication machinery of the cell. PCNA has been identified as a cofactor of DNA polymerase δ (6, 46, 55). Mercer et al. (39) demonstrated a down-regulation of PCNA mRNA and protein by wild-type p53. However, the mechanism of this regulation was not known since the possibility that p53 might affect the PCNA promoter activity was not examined.

Several viruses have mechanisms to target (and presumably inactivate) wild-type p53 by their transforming proteins. SV40 large T antigen, adenovirus 5 E1B, and E6 of human papillomavirus (HPV) types 16 and 18 bind specifically to p53 and either sequester it (large T, E1B) or promote its degradation (E6) (31, 33, 51, 52, 60). The effect of p53 on promoter activity of viruses has not been investigated in detail.

We studied the effect of wild-type and mutant human p53 expression on the activity of PCNA and several viral promoters fused to a chloramphenicol acetyltransferase (CAT) reporter gene. Expression of wild-type p53 correlated with a consistent and significant (6- to 76-fold) inhibition of reporter enzyme activity in HeLa cells. Significantly, mutants of p53 found in cancer cell lines exert this inhibitory effect on the promoter function in this assay. This suggests that the promoter inhibitory activity of p53 is crucial for its tumor suppressor activity.

## MATERIALS AND METHODS

**DNA plasmids.** Wild-type and mutant human p53 expression plasmids (generously provided by Arnold J. Levine) utilize the human cytomegalovirus (HCMV) major immedi-

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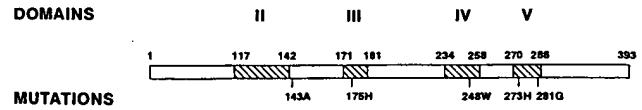


FIG. 1. Schematic representation of the p53 gene product. Conserved domains II to V are indicated by hatched areas. Positions of amino acid substitutions in the mutants that are used in this study are indicated below.

ate-early promoter-enhancer (−671 to +73) in the vector pHCMV-Neo-Bam (25). p53-cWT contains a wild-type p53 cDNA, while p53-c143A (Val to Ala at amino acid 143) and p53-c248W (Arg to Trp at amino acid 248) contain mutant p53 cDNAs (25). p53-175H (Arg to His at amino acid 175), p53-273H (Arg to His at amino acid 273), and p53-281G (Asp to Gly at amino acid 281) are mutant p53 cDNA-genomic chimeras, all containing introns 2 through 4 (25). The neomycin resistance gene was removed from all plasmids by treatment with *Hin*dIII and *Xba*I.

The CAT plasmids described here all contain the *Escherichia coli* CAT gene under the transcriptional control of the following promoters: PCNA (human PCNA promoter) (41); pSV2 (SV40 early promoter-enhancer) (22); CMV (HCMV major immediate-early promoter-enhancer) (11); HSV-1.TK (herpes simplex virus type 1 thymidine kinase promoter) (37); UL9 (HSV-1 UL9 gene promoter [12a]); RSV (Rous sarcoma virus 3′ long terminal repeat [LTR]) (12); HIV-1 (human immunodeficiency virus type 1 LTR) (43); and HTLV-I (human T-cell lymphotropic virus type I LTR) (53). The plasmids are designated promoter name.CAT. PCNA.CAT was generously provided by Gilbert Morris.

**Cell culture and transfection.** Human cervical carcinoma (HeLa) and monkey kidney (Vero) cells were obtained from the American Type Culture Collection and propagated in minimum essential medium containing 10% fetal calf serum and Dulbecco's minimum essential medium, respectively. Subconfluent cells were transfected by the calcium phosphate-DNA coprecipitation method with a dimethyl sulfoxide shock 4 h posttransfection (8, 23). In a typical experiment, $5 \times 10^6$ cells were cotransfected with 2.5 μg of a reporter gene construct and 5 μg of a p53 expression plasmid (or 5 μg of the expression vector without p53 sequences as a control). All transfection experiments were repeated several times.

**CAT assay.** Cells were harvested 48 h posttransfection and lysed by three successive cycles of freezing and thawing. Extracts were normalized for protein concentration and assayed for CAT enzyme activity (22). CAT activity was detected by thin-layer chromatographic separation of [$^{14}$C]chloramphenicol from its acetylated derivatives and quantitated by cutting out radioactive spots from the thin-layer chromatograph plate after autoradiography.

**Metabolic labeling and immunoprecipitation.** At 48 h after transfection with 20 μg of wild-type or mutant human p53 expression plasmids (or expression vector pHCMV-Bam), HeLa cells were incubated in methionine-free minimal essential medium for 20 min and subsequently metabolically labeled for 4 h with [$^{35}$S]methionine (ICN Tran[$^{35}$S]-label) at 70 μCi/ml in methionine-free minimal essential medium (49). Cells were lysed, and extract aliquots were immunoprecipitated with PAb421, a cross-species, carboxy-terminal-specific, anti-p53 monoclonal antibody (p53 Ab-1; Oncogene Science) (57), and protein A-agarose (Calbiochem). Immunoprecipitated proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and visualized by autoradiography.

## RESULTS

**Expression of wild-type and mutant human p53 proteins in transfected HeLa cells.** We used wild-type and mutant human p53-expressing clones for our analysis of the effect(s) of p53 on the function of various promoters. The mutants were c143A, 175H, c248W, 273H, and 281G, where capital letters indicate mutant amino acids and small c indicates cDNA clones. These mutants were chosen because they contain the frequently mutated amino acid residues found in tumors (26) (Fig. 1). These residues fall in or near four domains (II to V) which are highly conserved in vertebrate species (54).

To determine whether the mutants and wild-type proteins were expressed, we transfected HeLa cells with wild-type or mutant p53 expression plasmids or with expression vector pHCMV.Bam (vector alone) and metabolically labeled them with [$^{35}$S]methionine. Cell lysates were immunoprecipitated with PAb421, a cross-species, carboxy-terminal-specific, anti-p53 monoclonal antibody, and protein A-agarose. Immunoprecipitated proteins were separated by SDS-PAGE and visualized by autoradiography (Fig. 2). Transfection of HeLa cells with either wild-type or mutant p53 expression plasmids led to the specific immunoprecipitation of proteins migrating at an approximate molecular weight of 53,000 (indicated by an arrowhead), while transfection with the expression vector did not. Mutant p53 proteins appear to be expressed at higher levels than wild-type in transfected HeLa cells. This is consistent with the extended half-life of mutant p53 proteins (32). This effect would also be due to inhibitory effects exerted by wild-type p53 on the CMV promoter. Mutant proteins would not be as inhibitory, resulting in a higher level of expression. In the lanes containing mutant p53s (c143A, 175H, and 281G), a band at about 70 kDa is visible. This band may indicate complex formation between mutant p53s and the cellular heat shock protein 70 (25, 32). We do not know the identity of the 18-, 43-, and 200-kDa bands seen in all lanes, including vector alone. The immunoprecipitation results shown in Fig. 2 clearly indicate successful expression of wild-type and mutant human p53s in HeLa cells after transfection with the corresponding expression plasmids.

**Modulation of PCNA promoter activity by wild-type and**

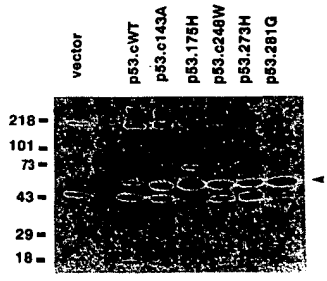


FIG. 2. Expression of wild-type and mutants of human p53 by transfection of expression plasmids into HeLa cells. HeLa cells were transfected with pHCMV.Bam expression vector alone and wild-type or mutant human p53 expression plasmid DNA; the proteins were then metabolically labeled with [$^{35}$S]methionine and immunoprecipitated with p53-specific monoclonal antibody as described in Materials and Methods. Immunoprecipitates were analyzed on an SDS-polyacrylamide gel. The arrowhead shows bands corresponding to p53. Numbers on left show sizes in kilodaltons.

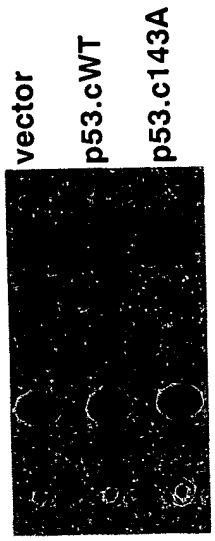


FIG. 3. Effect of wild-type and a mutant human p53 on the expression of PCNA.CAT in HeLa cells. Subconfluent HeLa cells were cotransfected with PCNA.CAT (2.5 μg) and pHCMV.Bam (vector alone) or pHCMV.Bam expressing either wild-type or a mutant p53 (143 V→A), using the calcium phosphate precipitation technique as described in Materials and Methods. At 48 h posttransfection, cells were harvested and a CAT assay was done as described in the text. Experiments were repeated several times with similar qualitative results; one representative example is shown.

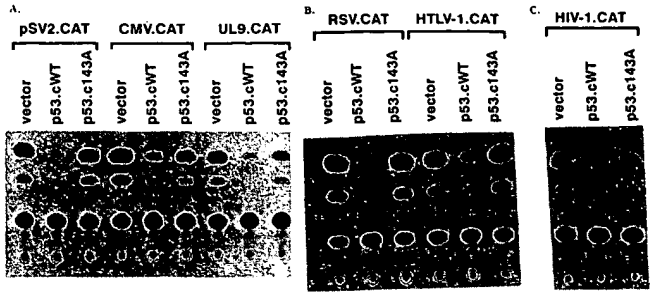


FIG. 4. Effect of wild-type and a mutant human p53 on the expression of viral promoter-CAT constructs in HeLa cells. The promoter-CAT constructs indicated (see text) were transfected separately into HeLa cells along with pHCMV.Bam (vector alone) or pHCMV.Bam expressing either wild-type or mutant p53 (143 V→A) as described in the legend to Fig. 3. For pSV2.CAT, 0.5 μg of the CAT plasmid was used with 5 μg of vector or p53 expression plasmid. All others were used as described in Materials and Methods.

**mutant human p53.** The PCNA gene encodes a nuclear protein that acts as an auxiliary factor of DNA polymerase δ and is presumably a part of the cellular replication machinery (55). It has been shown previously (39) that growth suppression induced by wild-type p53 protein is accompanied by a down-regulation of PCNA expression. Therefore, we were interested in determining whether wild-type p53 can inhibit the function of the PCNA promoter and, if so, whether a mutant p53 can exert the same effect. PCNA.CAT (41) was cotransfected into HeLa cells by the calcium phosphate precipitation technique as described in Materials and Methods with the pHCMV.Bam expression vector alone or with the plasmid expressing either the wild-type or the mutant (c143A) form of p53. After 48 h, CAT activity (PCNA promoter activity) was assayed in these cells. Wild-type p53 inhibited PCNA.CAT activity in transient assays by more than sixfold, while the mutant inhibited activity by one- to twofold (Fig. 3). Thus, the promoter inhibition is due to wild-type p53, and a mutation in the p53 gene destroys the inhibitory effect.

**Effect of expression of wild-type and mutant p53 on various viral promoters.** To analyze the effect of expression of wild-type and mutant p53 on various viral promoters, we used the following promoter-CAT constructs: SV40 early promoter (pSV2.CAT) (22), CMV early promoter-enhancer (CMV.CAT) (11), HSV-1 UL9 promoter (UL9.CAT) (13a), HIV-1 LTR (HIV.CAT) (43), RSV LTR (RSV.CAT) (12), and HTLV-I LTR (HTLV.CAT) (53). The promoter activities were determined by CAT assay after cotransfecting the respective promoter constructs with the pHCMV.Bam expression vector alone or with the plasmid expressing either the wild-type or a mutant (c143A) form of p53 into HeLa cells (Fig. 4; Table 1). The experiments were repeated several times with qualitatively similar results. Representative examples are shown in Fig. 4. All the promoters were inhibited significantly by the expression of wild-type p53. On the other hand, the mutant p53 had a relatively minor, if any, effect on expression of the various promoter-CAT constructs. In most of the cases, although the inhibition persisted with the mutant p53 (c143A), its extent was greatly reduced. In at least one case (HTLV-.CAT), the mutant actually stimulated the activity about 50%. This is not entirely surprising since recently Chin et al. (10) reported that the human multi-drug-resistant (*MDR*1) gene promoter is activated by another mutant p53 (175H).

All the promoters examined were inhibited by wild-type human p53, albeit to different extents. SV40 early promoter seems to be least affected under our assay conditions. To observe a significant extent of inhibition, we had to lower the pSV2.CAT concentration to 0.5 μg per transfection. The difference in the extent of inhibition by the same amount of wild-type p53 expression construct indicates that the observed promoter inhibition is possibly not an effect of general lethality caused by p53.

**Effect of expression of wild-type and various mutants of human p53 on HSV-1 TK gene promoter.** To determine the effect of other mutant p53 proteins on promoter activity, we tested wild-type and various mutants of human p53 with the HSV-1 TK gene promoter-CAT construct TK.CAT. The mutants of human p53 chosen for this study were described above and are as follows: p53-c143A, p53-175H, p53-c248W, p53-273H, and p53-281G. As shown in Fig. 5, TK promoter activity was inhibited most dramatically by wild-type p53, while the mutants inhibited to different extents. It is clear

TABLE 1. Inhibition of activity of different promoters by human wild-type (WT) p53 and mutant c143A relative to vector alone in HeLa cells

| Promoter | Activity relative to vector alone (%) | |
|---|---|---|
| | WT p53 | c143A p53 |
| PCNA | 15.9 | 58.8 |
| RSV LTR | 1.3 | 76.9 |
| HTLV-I LTR | 14.3 | 156.3 |
| HIV LTR | 2.3 | 47.6 |
| UL9 (HSV) | 2.8 | 31.2 |
| SV40 early promoter | 6.1 | 90.9 |
| CMV early promoter | 7.2 | 16.1 |

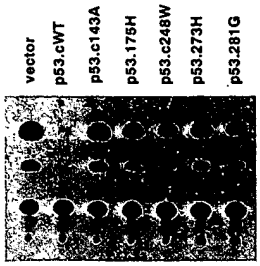


FIG. 5. Effect of expression of different mutant human p53s on the expression of HSV-1 TK promoter activity. HeLa cells were cotransfected with TK.CAT and pHCMV.Bam (vector alone) or pHCMV.Bam expressing either wild-type (cWT) or one of the mutant p53s: c143A (V to A at amino acid 143), 175H (R to H at amino acid 175), c248W (R to W at amino acid 248), 273H (R to H at amino acid 273), and 281G (D to G at amino acid 281) as described in the text. CAT assays were done as described in the text.

that all the mutants tested show a dramatic loss in mediating inhibition of the promoter. The failure of tumor suppression by these mutant p53 proteins correlates with the loss of the promoter inhibitory effect.

**Inhibition of activity of various promoters by wild-type human p53 in Vero cells.** To determine whether the p53-mediated promoter inhibition is cell type specific or is influenced by the expression of E6 of HPV 18 in the HeLa cell line, we chose also to use a monkey kidney cell line (Vero). Table 2 shows the percentage of acetylation of [$^{14}$C]chloramphenicol with various promoters in the presence and absence of wild-type human p53. The results indicate that in the Vero cell line (a nontransformed cell line) also, wild-type human p53 significantly inhibits various promoter activities (6- to 28-fold).

## DISCUSSION

The results described above show that overexpression of wild-type human p53 can exert an inhibitory effect on a variety of viral promoters as well as on the cellular PCNA promoter (6- to 76-fold, Table 1). Several other groups recently reported an inhibitory activity of p53 on different cellular promoters. Santhanam et al. (50) found that wild-type p53 inhibited the promoters for interleukin 6, c-*fos*, beta-actin, and the porcine major histocompatibility complex class I gene. Ginsberg et al. (21) described the inhibition of c-*fos*, beta-actin, p53, hsc70, and c-*jun* promoters, while Chin et al. (10) showed that the *MDR*1 gene promoter was inhibited by p53. Combining our results with those reported previously, it becomes clear that a wide variety of cellular and viral promoters are inhibited by wild-type human p53. In all the cases, mutant p53 proteins found in tumors were either less inhibitory or in some cases stimulatory (10) (Fig. 4) for the promoter function. The extent of inhibition by wild-type p53 appears to depend on the promoter tested (Table 1); for example, the CMV promoter (CMV.CAT) was not inhibited to the same extent as the HIV LTR promoter (HIV.CAT). The SV40 early promoter seems to be least affected under our assay conditions. To observe a significant extent of inhibition, we had to lower the pSV2.CAT concentration to 0.5 μg per transfection. The difference in the extent of inhibition by the same amount of wild-type p53-expressing construct indicates that the observed promoter inhibition is possibly not an effect of general lethality caused by p53. Also, different mutants of p53 have different quantitative effects on promoter inhibition (Fig. 5; Table 3). Despite these differences, it is both interesting and significant that such a wide variety of viral promoters are inhibited by p53. What effect endogenous p53 has on these promoters in the course of viral infection is not known. It remains to be seen whether all these viruses may have molecular mechanisms to circumvent p53 inhibition. This may represent a unique strategy to allow viral replication not previously defined for nononcogenic viruses, whereas alteration of p53 by tumor viruses such as SV40, adenovirus, and HPV 16 and 18 has been established (31, 33, 51, 52, 60).

TABLE 2. Inhibition of different promoters by human wild-type p53 relative to vector alone in Vero cells

| Promoter | Activity relative to vector alone (%) | Fold inhibition |
|---|---|---|
| PCNA | 15.4 | 6.5 |
| RSV LTR | 3.5 | 28.6 |
| HIV LTR | 10.3 | 9.7 |
| UL9 (HSV) | 11.1 | 9.0 |
| CMV | 12.5 | 8.0 |

TABLE 3. Inhibition of HSV-1 TK promoter by human wild-type p53 and different mutants

| p53 | Activity relative to vector alone (%) |
|---|---|
| cWT | 1.1 |
| c143A | 66.7 |
| 175H | 37.0 |
| c248W | 14.5 |
| 273H | 37.0 |
| 281G | 21.2 |

Most of our experiments were performed in HeLa cells, which are known to have the E6 protein of HPV 18. Since HPV 18 E6 protein is known to interact with p53, a possibility remains that the data observed were influenced by this interaction. However, we also observed significant promoter inhibition in Vero cells (a nontransformed cell line) (Table 2). This suggests that the promoter inhibition is probably because of p53 alone.

Because p53 possesses the promoter inhibitory activity, it is possible that at least one of the mechanisms by which wild-type p53 inhibits cellular proliferation is by inhibiting cellular promoters. This is based on the assumption that p53 directly inhibits transcriptional activity. This remains to be determined by using in vitro transcription systems. However, the fact that the inhibitory effect of p53 is exerted on a wide variety of promoters, both cellular and viral, suggests that p53 probably affects one or more of the common generalized transcription factors or that it binds to promoter sequences nonspecifically and inhibits transcription. At this stage, we should also be aware of the possibility that the observed promoter inhibition is an effect of wild-type p53 when it is overexpressed. Under normal conditions, such a high concentration of p53 is not expected. However, it is not difficult to imagine that at a certain point in the cell cycle, local effective concentration of p53 may rise high enough to modulate cellular promoter activities.

p53 remains an extremely important and intriguing molecule. It has been demonstrated that purified wild-type p53 can bind to cellular DNA (30) as well as the SV40 early promoter region (4). It has also been shown that it can

function as a transcriptional activator when expressed as a chimera with the GAL4 DNA-binding domain on promoters with GAL4 DNA-binding sites (17, 47). The same molecule may function as an activator as well as an inhibitor of transcription. It is tempting to speculate that while p53 acts as a generalized inhibitor of transcription, it could activate certain promoters where it can bind effectively. We have observed that it requires a relatively higher concentration of p53 plasmid to inhibit the SV40 early promoter, which has p53-binding sites (4). One can speculate that p53 may exert its tumor suppressor function in several ways. First, under certain conditions, p53 may inhibit genes required for progression through the cell cycle. The inhibition of the PCNA promoter demonstrated in this study supports this mechanism. In addition, p53 may also activate expression of genes involved in the regulation of normal cell cycle progression. This regulation of expression may require the presence of p53-binding sites as *cis*-acting factors at the target gene. A possibility also exists that p53 activates the production of a factor that interacts with the transcription machinery and inhibits gene expression. Thus, p53 may act as a central factor in controlling the dynamic pattern of gene expression required for maintenance of a normal cell cycle. Both the tumor suppressor gene products RB and p53 have cellular antiproliferative activity. In one way, at least, they have a similar biochemical function—inhibition of transcription. It has been suggested that at least one mechanism by which RB may inhibit specific transcriptional activity is by complexing with the transcription factor E2F (7, 42). While it is not yet clear how p53 exerts its effect, similarity in biochemical function is an intriguing common theme.

## ACKNOWLEDGMENTS

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